Effects of glibenclamide on glycylsarcosine transport by the rat peptide transporters PEPT1 and PEPT2

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1 Glibenclamide is a widely used sulphonylurea for the treatment of non-insulin-dependant diabetes mellitus (NIDDM). This agent has been reported to inhibit the activities of various ion channels and transporters. In the present study, we examined the effects of glibenclamide on the function of the H^+/peptide cotransporters PEPT1 and PEPT2 by using stable transfectants.

2 Uptake of [^{14}C]-glycylsarcosine, a typical substrate for peptide transporters, by PEPT1- or PEPT2-expressing transfectant was inhibited by glibenclamide as well as other sulphonylureas including tolbutamide.

3 Kinetic analysis revealed that the inhibition by glibenclamide was noncompetitive. Dixon plot analyses showed that the K_i values of this agent were 25 and 7.8 μM for PEPT1 and PEPT2, respectively.

4 Glibenclamide did not inhibit Na^+-coupled alanine and α-methyl-D-glucoside transport, suggesting that the inhibitory effects of glibenclamide on peptide transporters were not due to nonspecific interactions.

5 There was little uptake of [^{3}H]-glibenclamide by PEPT-expressing transfectants as compared to mock-transfected cells, suggesting that glibenclamide was not a substrate for these peptide transporters.

6 In summary, glibenclamide inhibited the [^{14}C]-glycylsarcosine transport by PEPT1 and PEPT2 in a noncompetitive fashion, although glibenclamide *per se* was not transported through these transporters. These findings would provide important information for clinical, physiological and biochemical aspects of peptide transporters.

Keywords: Glibenclamide; peptide transporters; sulphonylureas; drug interaction; noncompetitive inhibition

Abbreviations: αMG, α-methyl-D-glucoside; CFTR, the cystic fibrosis transmembrane conductance regulator; DMSO, dimethyl sulphoxide; MES, 2-(N-morpholino)ethanesulphonic acid; NIDDM, non-insulin-dependent diabetes mellitus

Introduction

Glibenclamide is a second-generation sulphonylurea which has been widely used in the management of non-insulin-dependent diabetes mellitus (NIDDM). Glibenclamide induces insulin secretion by blocking ATP-sensitive potassium channels (K_{ATP} channel) in pancreatic β-cells (Panten *et al.*, 1996; Babenko *et al.*, 1998). This agent is also known to inhibit the activities of several ion channels and transporters, such as the cystic fibrosis transmembrane conductance regulator (CFTR) and the hepatic bile acid transporter (Fückel & Petzinger, 1992; Sheppard & Welsh, 1992; Kirk *et al.*, 1993; Sheppard & Robinson, 1997). Therefore, it is assumed that glibenclamide not only exhibits its own pharmacological action but also causes unexpected effects by modulating these membrane proteins.

H^+-coupled peptide transporters, which are localized at brush-border membranes of epithelial cells, mediate the absorption of various peptide-like drugs, as well as that of di- and tripeptides. As peptide transporters are primarily expressed in the small intestine and kidney, the pharmacokinetic profiles of peptide-like drugs, i.e. their absorption rates and biological half-lives, can be affected by the transport activities of peptide transporters. Two distinct peptide transporters, intestinal PEPT1 and renal PEPT2,

have been cloned and characterized (Leibach & Ganapathy, 1996; Daniel & Herget, 1997; Inui & Terada, 1999). Functional studies have confirmed that a wide variety of pharmacologically active peptide-like drugs such as β-lactam antibiotics (Saito *et al.*, 1995; Terada *et al.*, 1997a) and the anticancer agent bestatin (Saito *et al.*, 1996) are substrates of PEPT1 and PEPT2. Furthermore, the structural requirements of substrates for PEPT1 and PEPT2 were recently re-evaluated, and it was shown that PEPT1 and PEPT2 recognize various nonpeptidic substrates (Börner *et al.*, 1998; Döring *et al.*, 1998a,b; Ganapathy *et al.*, 1998; Han *et al.*, 1998; Temple *et al.*, 1998).

As glibenclamide is administered orally, we hypothesized that the functions of some intestinal transporters might be affected by this agent crossing the intestinal epithelial cells. The intestinal peptide transporter, PEPT1, attracted our attention because of its important role in the therapeutic efficacy of peptide-like drugs. Recent findings of the broad substrate specificity of PEPT1 also led us to examine the interaction of glibenclamide with this transporter, although glibenclamide is a nonpeptidic compound. Furthermore, comparison of the interaction between PEPT1 and PEPT2 may be important in characterizing the biological aspects of peptide transporters. Therefore, in the present study, we examined the interactions of glibenclamide with peptide transporters using stable transfectants expressing rat PEPT1 or rat PEPT2.

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Methods

Materials

[^{14}C]-glycylsarcosine (1.78 GBq mmol^{-1}) was obtained from Daiichi Pure Chemicals Co., Ltd. (Ibaraki, Japan). [^{3}H]-glibenclamide (1.85 Tbq mmol^{-1}) and [^{3}H]-alanine (2.07 Tbq mmol^{-1}) were purchased from Amersham International plc. (Buckinghamshire, U.K.). Methyl-α-D-[^{14}C(U)]-glucopyrano-side (α-methyl-D-glucoside (αMG)) (9.66 Gbq mmol^{-1}) was supplied by Moravek Biochemicals Inc. (Brea, CA, U.S.A.). Glycylsarcosine, tolbutamide and chlorpropamide were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glibenclamide was purchased from Wako Chemical Co. (Kyoto, Japan). All other chemicals used were of the highest purity available. Figure 1 shows the chemical structures of the sulphonylureas used.

Cell culture

Parental LLC-PK$_1$ cells were obtained from the American Type Culture Collection (ATCC CRL-1392). The LLC-PK$_1$ cells transfected with rat PEPT1 cDNA (LLC-rPEPT1), with rat PEPT2 cDNA (LLC-rPEPT2) and with empty vector (LLC-pBK) were prepared and cultured as described previously (Terada *et al.*, 1997a,b).

Uptake studies by cell monolayers

Cellular uptake of [^{14}C]-glycylsarcosine was measured by monolayers grown in 35-mm plastic dishes as described previously (Terada *et al.*, 1997b). The incubation medium contained (mM): NaCl, 145; KCl, 3; CaCl$_2$, 1; MgCl$_2$, 0.5; D-glucose, 5, and 2-(N-morpholino)ethanesulphonic acid (MES), 5 (pH 6.0) or HEPES, 5 (pH 7.4). Sulphonylureas were dissolved in dimethyl sulphoxide (DMSO) and the final concentration of DMSO in the incubation medium was 1.0%. The protein contents of cell monolayers solubilized in 1 M NaOH were determined by the method of Bradford (1976) using a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA, U.S.A.) with bovine γ-globulin as the standard.

Statistical analysis

Data were analysed statistically by non-paired *t*-test or one-way analysis of variance followed by Scheffé's test when multiple comparisons were needed.
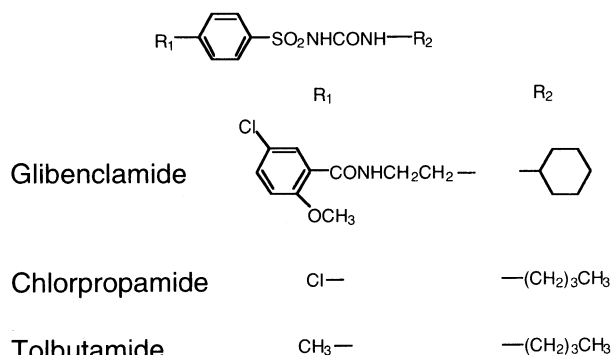
Results

First, we examined the effects of various sulphonylureas on the uptake of [^{14}C]-glycylsarcosine, a typical substrate for peptide transporters, by rat PEPT1- or rat PEPT2-expressing transfectants (LLC-rPEPT1 or LLC-rPEPT2 cells). As shown in Figure 2, [^{14}C]-glycylsarcosine uptake by LLC-rPEPT1 and LLC-rPEPT2 cells was markedly inhibited by glibenclamide at 50 μM. Other sulphonylureas, i.e. tolbutamide and chlorpropamide, also significantly inhibited [^{14}C]-glycylsarcosine uptake by both transfectants at the concentration of 1 mM. The solvent used for sulphonylureas, DMSO, showed no effect on [^{14}C]-glycylsarcosine uptake (data not shown).

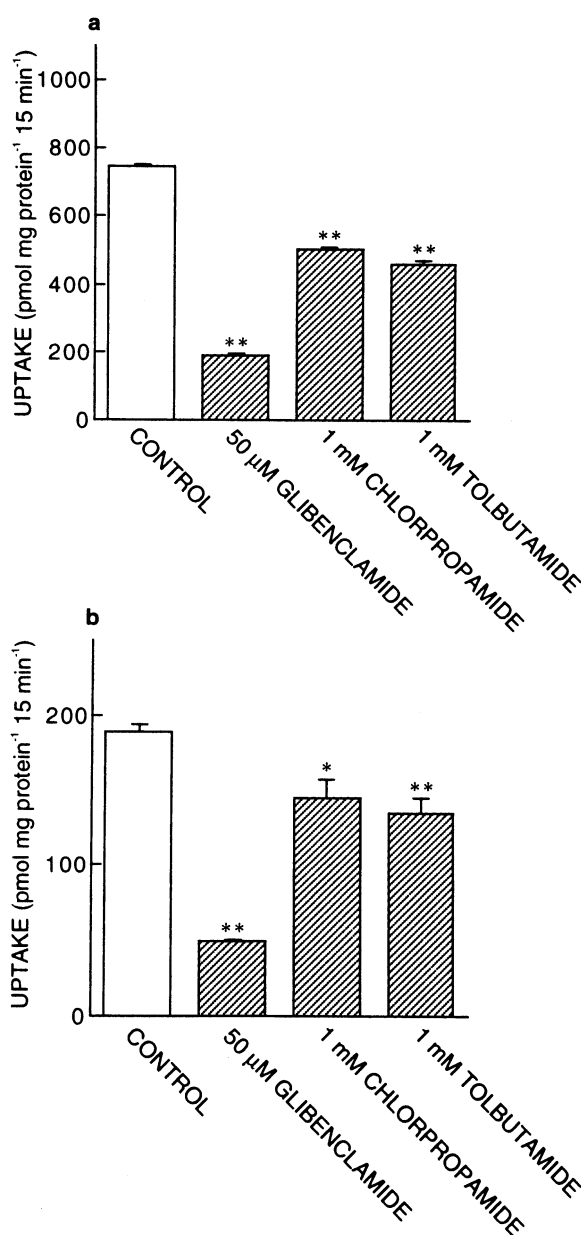To investigate the kinetics of the inhibitory effects of glibenclamide on [^{14}C]-glycylsarcosine uptake, the concentra-

Figure 2 Effects of various sulphonylureas on [^{14}C]-glycylsarcosine uptake by LLC-rPEPT1 (a) and LLC-rPEPT2 cells (b). The cell monolayers were incubated with incubation medium containing [^{14}C]-glycylsarcosine (20 μM, pH 6.0) for 15 min at 37°C in the absence or presence of each inhibitor. Each column represents the mean± s.e.mean of six monolayers from two separate experiments. *$P<0.05$, **$P<0.01$, significantly different from control.

Figure 1 Chemical structures of sulphonylureas.

tion dependence of [^{14}C]-glycylsarcosine uptake was examined in the absence or presence of this agent (Figure 3). Specific uptake was calculated by subtracting the nonspecific uptake, which was estimated in the presence of excess unlabelled dipeptide, from the total uptake. Kinetic parameters were calculated according to the Michaelis-Menten equation using nonlinear least squares regression analysis. The presence of glibenclamide markedly decreased the maximal velocities (V_{max} values for control versus those in the presence of glibenclamide: 74 versus 27 for PEPT1; 1.0 versus 0.24 for

PEPT2, nmol mg protein^{-1} 15 min^{-1}). On the other hand, the apparent affinities were comparable (K_M values for control versus those in the presence of glibenclamide: 1.8 versus 1.6 for PEPT1; 0.096 versus 0.078 for PEPT2, mM). These results suggested that glibenclamide noncompetitively inhibited [^{14}C]-glycylsarcosine uptake by both PEPT1 and PEPT2.

To determine the inhibition constant (K_i) values of glibenclamide, Dixon plot analyses were performed. As illustrated in Figure 4, the plots were linear and crossed on the abscissa in both transfectants, suggesting that glibenclamide noncompetitively inhibited [^{14}C]-glycylsarcosine uptake.
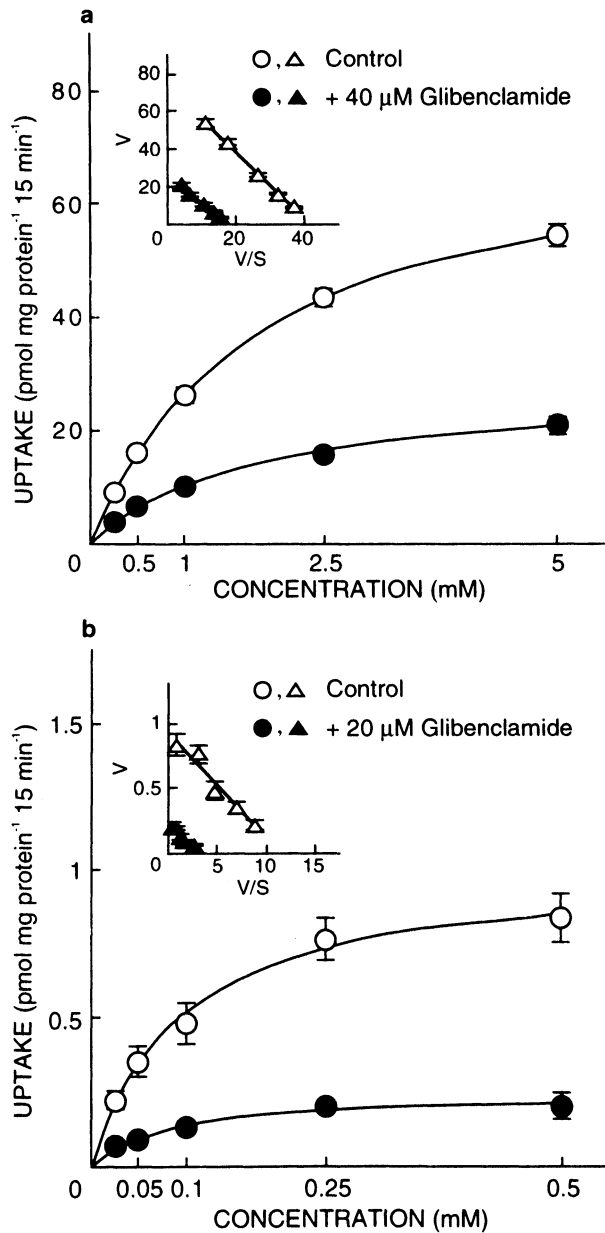
Figure 3 Concentration dependence of [^{14}C]-glycylsarcosine uptake in the absence or presence of glibenclamide in LLC-rPEPT1 (a) and LLC-rPEPT2 cells (b). The cell monolayers were incubated with incubation medium containing various concentrations of [^{14}C]-glycylsarcosine (pH 6.0) for 15 min at 37°C. Nonspecific uptake was evaluated by measuring [^{14}C]-glycylsarcosine uptake in the presence of 50 mM glycylleucine, and the results are shown after correction for the nonsaturable component. Inset, Eadie-Hofstee plots of glycylsarcosine uptake after correction for the nonsaturable component. Each point represents the mean±s.e.mean of six monolayers from two separate experiments. When the error bars are not shown, they are smaller than the symbol.
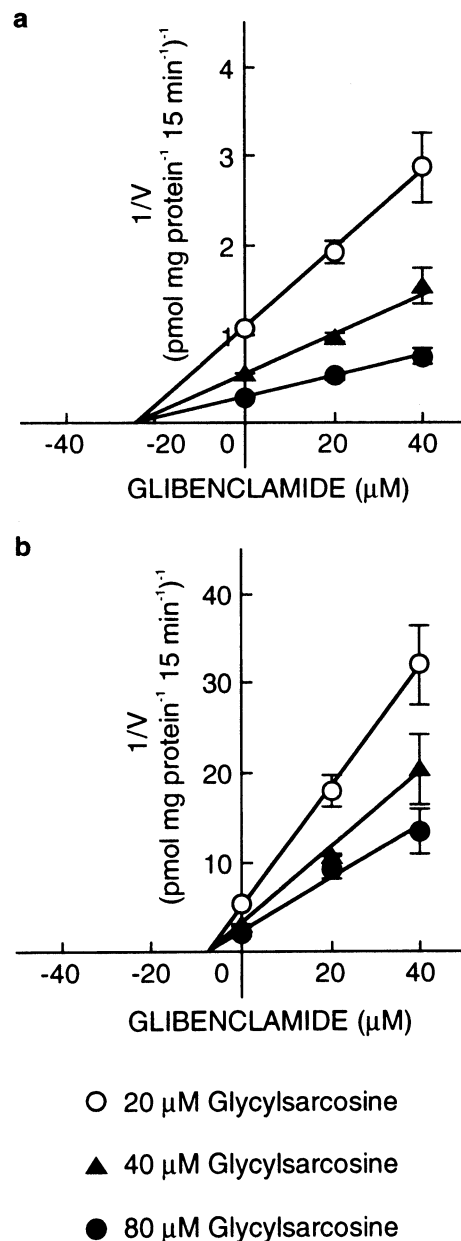
Figure 4 Dixon plots showing the inhibitory effects of glibenclamide on [^{14}C]-glycylsarcosine uptake by LLC-rPEPT1 (a) and LLC-rPEPT2 cells (b). The cell monolayers were incubated for 15 min at 37°C with incubation medium containing [^{14}C]-glycylsarcosine at pH 6.0. Data were corrected for the nonsaturable component by measuring [^{14}C]-glycylsarcosine uptake in the presence of 20 mM glycylleucine. Each point represents the mean±s.e.mean of six monolayers from two separate experiments. When the error bars are not shown, they are smaller than the symbol.

The K_i values calculated from the points of intersection were as follows: 25 μM for PEPT1 and 7.8 μM for PEPT2.

Next, we examined whether glibenclamide affected the H^+ gradient as the driving force of PEPT1 and PEPT2. As shown in Figure 5, [^{14}C]-glycylsarcosine uptake at both pH 6.0 (with a H^+ gradient) and pH 7.4 (without a H^+ gradient) was inhibited by glibenclamide in LLC-rPEPT1 and LLC-rPEPT2 cells.

Parental LLC-PK$_1$ cells were reported to show Na$^+$-dependent and carrier-mediated transport for alanine (Kimmich et al., 1994) and α-methyl-D-glucoside (αMG) (Bennett & Kimmich, 1996). We found that uptake of [^{3}H]-alanine and [^{14}C]-αMG by both transfectants was decreased under Na$^+$-free conditions, and was inhibited in the presence of either unlabelled excess alanine or αMG (data not shown). We subsequently examined the effect of glibenclamide on [^{3}H]-alanine and [^{14}C]-αMG uptake. As shown in Figure 6, glibenclamide did not inhibit [^{3}H]-alanine or [^{14}C]-αMG uptake by either transfectant.

Finally, we examined whether glibenclamide was transported by peptide transporters. In a preliminary study, we found that the rate of [^{3}H]-glibenclamide uptake by LLC-rPEPT1 cells was linear during the first 1 min of incubation. Therefore, we examined [^{3}H]-glibenclamide uptake by LLC-pBK, LLC-rPEPT1 and LLC-rPEPT2 cells during a 30-s incubation period. As shown in Table 1, there was no increase in [^{3}H]-glibenclamide uptake by LLC-rPEPT1 or LLC-rPEPT2 cells compared to LLC-pBK cells. In addition, 20 mM glycylsarcosine did not show an inhibitory effect on [^{3}H]-glibenclamide uptake. These results suggested that glibenclamide was not transported via peptide transporters.

Discussion

In the present study, we clearly demonstrated that glibenclamide is a potent inhibitor of the peptide transporters, PEPT1 and PEPT2, although glibenclamide itself was not a substrate for these transporters. Other sulphonylureas,
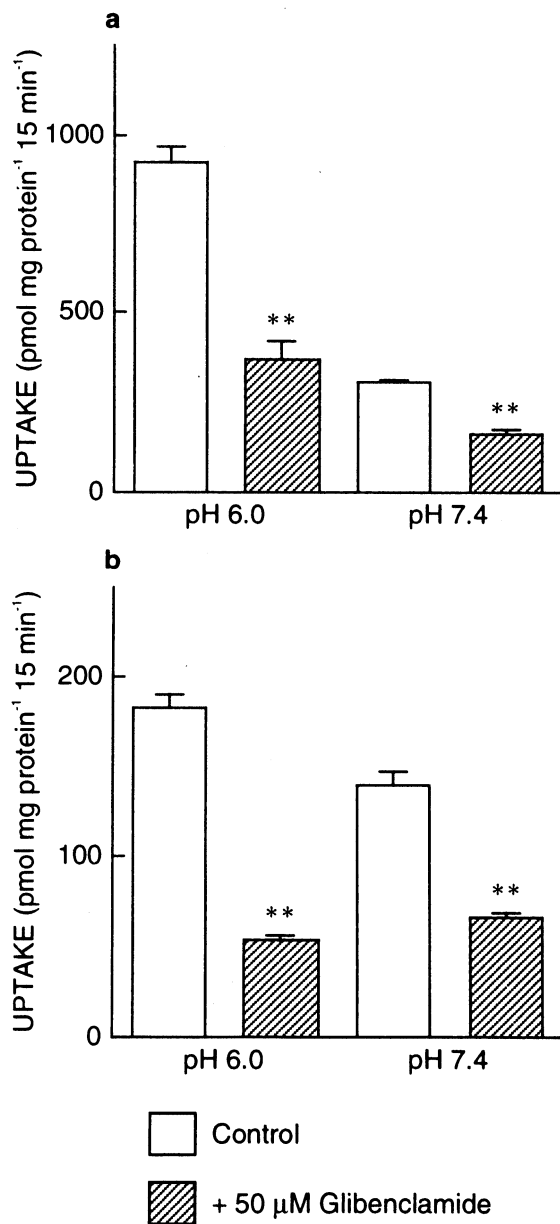
Figure 5 Effects of glibenclamide on [^{14}C]-glycylsarcosine uptake at pH 6.0 or pH 7.4 by LLC-rPEPT1 (a) and LLC-rPEPT2 cells (b). The cell monolayers were incubated with [^{14}C]-glycylsarcosine (20 μM) at pH 6.0 or pH 7.4 in the absence or presence of 50 μM glibenclamide for 15 min at 37°C. Each column represents the mean±s.e.mean of six monolayers from two separate experiments. **$P < 0.01$, significantly different from control.
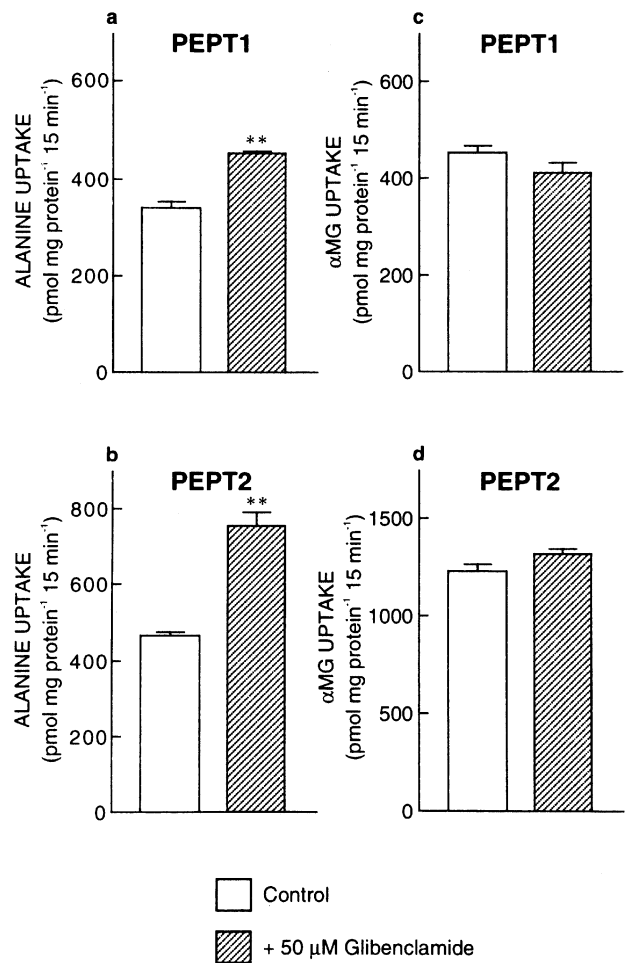
Figure 6 Effects of glibenclamide on [^{3}H]-alanine (a, b) and [^{14}C]-αMG (c, d) uptake by LLC-rPEPT1 and LLC-rPEPT2 cells. The cell monolayers were incubated with incubation medium containing [^{3}H]-alanine (20 μM, pH 6.0) or [^{14}C]-αMG (100 μM, pH 6.0) in the absence or presence of 50 μM glibenclamide for 15 min at 37°C. Each column represents the mean±s.e.mean of six monolayers from two separate experiments. **$P < 0.01$, significantly different from control.

Table 1 [^{3}H]-glibenclamide uptake by LLC-pBK, LLC-rPEPT1 and LLC-rPEPT2 cells

Transfectants	*Uptake* (pmol mg protein^{-1} 30 s$^{-1)}$)	
	Control	*Glycylsarcosine* (20 mM)
LLC-pBK	1.2 ± 0.03	1.2 ± 0.03
LLC-rPEPT1	1.3 ± 0.07	1.2 ± 0.01
LLC-rPEPT2	0.9 ± 0.02	0.8 ± 0.02

The cell monolayers were incubated with incubation medium containing [^{3}H]-glibenclamide (20 nM, pH 6.0) in the absence or presence of 20 mM glycylsarcosine for 30 s at 37°C. Each value represents the mean ± s.e.mean of six monolayers from two separate experiments.

tolbutamide and chlorpropamide, also inhibited the transport activities of PEPT1 and PEPT2. The calculated K_i value of glibenclamide was 25 μM for intestinal PEPT1, one of the highest affinities to this transporter reported to date (Terada *et al.*, 1997b; Ganapathy *et al.*, 1998). These findings have not only significant nutritional but also clinical implications, i.e. drug–drug interactions at the intestinal absorption level are possible. When glibenclamide is coadministered with peptide-like drugs such as oral β-lactam antibiotics to NIDDM patients, there is a possibility that the oral bioavailabilities of peptide-like drugs may be reduced. Therefore, our findings provide important insight into the therapeutic efficacy of such regimens as coadministration of sulphonylureas and peptide-like drugs.

The primary physiological role of peptide transporters is to mediate the transport of small peptides derived from dietary protein. However, recent investigations have indicated the novel physiological and pharmacological aspects of peptide transporters. For example, Döring *et al.* (1998a) demonstrated that delta-aminolevulinic acid, a precursor of porphyrin synthesis, was transported *via* PEPT1 and PEPT2 and suggested that it may be useful for photodynamic therapy. Merlin *et al.* (1998) showed that the intestinal PEPT1 transported formyl-Met-Leu-Phe, a major peptide neutrophil chemotactic factor, and suggested that modulating PEPT1 activity could influence states of intestinal inflammation. It is noted that glibenclamide is not administered transiently because NIDDM is a chronic disease. Therefore, the long-term administration of glibenclamide may affect physiological functions such as porphyrin metabolism and intestinal inflammation. Furthermore, PEPT2 was demonstrated to be expressed in a variety of tissues such as the lung, brain and mammary gland and it was suggested that this transporter may participate in as yet unknown physiological processes (Saito *et al.*, 1996; Döring *et al.*, 1998a). Therefore, it is important to evaluate the effects of long-term administration of glibenclamide on the functions of peptide transporters.

The inhibitory effect of glibenclamide was characterized kinetically as noncompetitive. Several mechanisms of this inhibition were considered. First, the noncompetitive inhibition might occur due to lipophilic nonspecific binding to membrane proteins because glibenclamide was reported to show a high serum protein binding ratio (99%) (Panten *et al.*, 1989). However, these possibilities were excluded because glibenclamide did not show an inhibitory effect on Na$^+$-coupled alanine and αMG transport. The data suggested that the inhibitory effect of glibenclamide on peptide transporters was not evoked by a nonspecific interaction. In fact, glibenclamide showed a stimulatory rather than an inhibitory effect on alanine uptake, although the reasons for this are unclear. Second, glibenclamide could affect the H$^+$ gradient or the membrane potential, which are the driving forces for peptide transporters. This was also unlikely, because glibenclamide inhibited [^{14}C]-glycylsarcosine uptake in the absence of a H$^+$ gradient, and Na$^+$-coupled αMG transport, which was clearly dependent on the membrane potential (Bennett & Kimmich, 1996), was not inhibited by glibenclamide. These results suggested that glibenclamide did not affect the driving force of peptide transporters. Third, it is possible that glibenclamide may bind to the transporter proteins at sites other than the substrate binding sites. Because glibenclamide has a sulphonylurea structure and an amide bond, these structures may be possible interaction sites with PEPTs. Recently, Akarawut *et al.* (1998) demonstrated that quinapril, an angiotensin converting enzyme inhibitor, noncompetitively inhibited glycylsarcosine uptake by PEPT2 using rabbit renal brush-border membrane vesicles, and suggested that it bound to a site distinct from that of the glycylsarcosine binding site. Although it is unclear whether glibenclamide and quinapril share a common biding site, these agents will be useful compounds to investigate the transport mechanisms of peptide transporters.

In conclusion, we found that the glibenclamide was a noncompetitive inhibitor of peptide transporters, although this agent itself is not a substrate for these transporters. To our knowledge, this is the first demonstration that sulphonylureas, especially glibenclamide, can inhibit the transport activities of peptide transporters. This agent should be a useful compound to examine the transport mechanism of peptide transporters. In addition, investigation of the interaction of sulphonylureas with peptide transporters may provide appropriate information for clinical usage of these drugs.

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